

Research Article

Bacterial Endo-Symbiont Inhabiting *Tridax procumbens* L. and Their Antimicrobial Potential

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Bacterial symbionts inhabiting *Tridax procumbens* L. were screened for antimicrobial potential with the aim to isolate potent bacteria bearing significant activity against test pathogens. The selected isolate was subjected to large scale fermentation to extract antimicrobial metabolite. The organic phase was reduced under vacuum pressure and crude ethyl acetate extract (10 mg/mL) was evaluated for antimicrobial activity against panel of test pathogens. The antibacterial activity was measured as a zone of inhibition and compared with standard antibiotics, gentamicin and tetracycline. Similarly, antifungal activity was compared with miconazole and bavistin. Significant activity was conferred against *Shigella flexneri* (MTCC 731) with 27 ± 1.5 mm zone across the disc. Partially, purification of antimicrobial metabolite with TLC-bioautography and HPLC resulted in active fraction bearing activity at R_f 0.65 and eluting between 4 and 5 retention times. The obtained results are promising enough for future purification and characterization of antimicrobial metabolite. Thus, the study attributes to the growing knowledge on endophytes as one of the rich sources of antimicrobial potentials.

1. Introduction

In recent years, microbial entities inhabiting medicinal plants are considered to be one of the rich novel sources of bioactive compounds bearing activities. In view of the ongoing efforts to combat drug resistant microbial pathogens, scientific communities are focusing on these microbial symbionts. It is well documented that every plant species is reported to inhabit diverse consortium of microbial symbionts which are known as endophytes [1]. These endophytes reside inside healthy tissues of plants and secrete bioactive metabolites bearing biological activities like antimicrobial, antitumor, antidiabetic, anti-inflammatory, antioxidant, and so forth [2]. Perusal of literatures confers the majority of studies conducted on fungal endophytes compared to bacterial endophytes which are known to be one of the dominant microbial communities

colonizing the inner tissues [3]. Owing to these facts, the present study aims to screen bacterial endophytic symbionts from medicinal *Tridax procumbens* L. The selection of plant was carried out based on the traditional knowledge and prior investigation on endophytes. Earlier study demonstrates antibacterial activity of crude extract from endophytes *Colletotrichum* sp. and *Alternaria* sp. isolated from *Tridax procumbens* L. [4]. *Tridax procumbens* L. is reported to possess significant medicinal value and is used as medicine in developing countries by tribal community for its wound healing properties. Studies also envision *Tridax procumbens* L. for antioxidant, antimicrobial, hepatoprotective, anti-inflammatory, and anticancerous properties [5]. Hence, the present investigation attributes to the growing scientific knowledge on endophytes as one of the merited microbial sources towards secreting antimicrobial metabolites.

2. Materials and Methods

2.1. Sample Collection. The plant material (*Tridax procumbens* L.) samples were collected from “Chamundi Hills” reserve forest (12°15'34"–12°17'5" north to 76°39'63"–76°42'02" east) that comprises an isolated range of hillocks ranging in altitude from 861 to 944 m above sea level, surrounding a plateau that is 1,024–1,057 m high with a periphery of 11 km, situated southeast at Mysore city, Karnataka, Southern part of India. Fresh and healthy samples were collected in a sterile polythene bags and brought to the laboratory and processed within 24 h. The selected plant was identified, authenticated, and deposited at the Department of Studies in Botany, University of Mysore, with accession number 721. The collected plants were thoroughly washed with tap water to remove soil and dirt particles and then immersed in double distilled water containing 50 µg/mL of cycloheximide for 60 min to suppress the growth of fungal endophytes [3].

2.2. Surface Sterilization for Strains Isolation. Stem, leaves, and root segments of *T. procumbens* were subjected to surface sterilization under aseptic condition by sequential steps of immersion in 3.15% sodium hypochlorite for 5 min, followed by ethanol 70% for 30 sec. Later, the outer tissue of surface sterilized stem, roots, and leaves were excised with sterilized scalpel into 0.5–1.0 cm tissue blocks and placed onto nutrient agar supplemented with 250 µg/mL of cycloheximide and incubated till bacterial endophytic colonies were visible. To confirm that the surface disinfection process was successful and to verify that there is no biological contamination from the surface during the surface sterilization protocol, sterility checks were carried out for each step to monitor the effectiveness by plating 0.1 mL of final rinse onto nutrient plates which served as control [3, 6].

2.3. Primary Screening of Endophytic Bacterial Isolates for Antimicrobial Activity

2.3.1. Test Strains. Eleven different strains of human and phytopathogenic microorganism, namely, *Bacillus subtilis* (MTCC 121), *Escherichia coli* (MTCC 7410), *Salmonella typhi* (MTCC 733), *Shigella flexneri* (MTCC 731), *Staphylococcus aureus* (MTCC 7443), *Staphylococcus epidermidis* (MTCC 435), *Xanthomonas campestris* (MTCC 7908), and *Candida albicans* (MTCC 183), were obtained from IMTECH-MTCC Chandigarh, India. *Fusarium verticillioides*, *Aspergillus niger*, and *Aspergillus flavus* were procured from DANIDA Laboratory, Mysore.

2.4. Primary Screening of Antimicrobial Potential. Antibacterial activity was evaluated by agar overlay method. Endophytic isolates were point-inoculated and incubated for 3 days at 37°C. Later, colonies were inactivated by inverting the upper lid with 1–5 mL chloroform for 40 mins. The inactivated colonies were overlaid with 5 mL of sloppy soft agar with 0.65% nutrient agar that had been inoculated with the test organisms. Zones of inhibition around the colonies were recorded after 24 h of incubation at 37°C. Endophytic

bacteria exhibiting activity were further subjected to scale production to extract the bioactive metabolites [7]. Similarly, antifungal activity was performed by dual culture method [8]. Endophytic isolate was streaked perpendicularly onto the potato dextrose agar medium, wherein agar plugs taken from an actively growing fungal culture were placed across the streak of bacterial isolate. Simultaneously, a control plate was maintained without any streaking of isolate and plates were incubated at room temperature for 3–4 days and observed for suppression of fungal growth.

2.5. Large Scale Fermentation of Potent Isolate. Based on the results from preliminary screening, endophyte bearing antimicrobial potential was subjected to large scale fermentation. Actively growing colony was aseptically inoculated into 1-litre Erlenmeyer flasks and incubated at 32°C with 120 rpm (rotation per minute) on rotary incubatory shaker. The samples were periodically monitored for increase in optical density at 600 nm with Shimadzu double beam spectrophotometer (Shimadzu Corp., Kyoto, Japan) [9].

2.6. Extraction and Analytical Detection of Metabolite. The supernatant obtained from fermentation of active isolate was extracted with ethyl acetate in the ratio 1:1 (v:v) and the organic phase was reduced under vacuum pressure at 40°C until dryness. The obtained dry mass was weighed and dissolved in minimal amount of ethyl acetate to obtain crude extract at concentration 10 mg/mL for further studies.

2.7. Evaluation of Antimicrobial Activity

2.7.1. Disc Diffusion Assay. Antibacterial activity was determined using the disc diffusion method. In brief, prewarmed MHA (Mueller-Hinton agar) plates were seeded with 10⁶ CFU (colony forming unit) suspensions of test bacteria and 50 µL crude ethyl acetate extract at 10 mg/mL (milligram/millilitre) was impregnated onto sterile paper discs (6 mm diameter) and placed on the surface of inoculated agar plates in triplicate. Plates were incubated at 37°C for 24 h. Antibacterial activity was measured as the diameter of the zone of inhibition across the disc. The results were interpreted and validated using ethyl acetate as negative control, and gentamicin at 1 mg/mL concentration (for antibacterial) and Bavistin at 1 mg/mL concentration (for antifungal) were used as positive control [10].

2.7.2. Bioautography. The active fraction bearing antimicrobial potential was determined with bioautography assay. Crude extract was partially purified using TLC plates with mobile phase ethyl acetate and methanol at a ratio 1:1. The developed TLC plates were overlaid with 1–2 mm layer of soft medium BHI (Brain Heart Infusion agar 0.6%), supplemented with 0.1% (w/v) 2,3,5-triphenyltetrazolium chloride (tetrazolium red) and previous seeded test organisms at a final concentration of 10⁷ CFU/mL. The plates were placed in a sterile tray, sealed to prevent the thin agar layer from drying, and incubated at 37°C for 24 hrs (hours); the plates were run in duplicate [11].

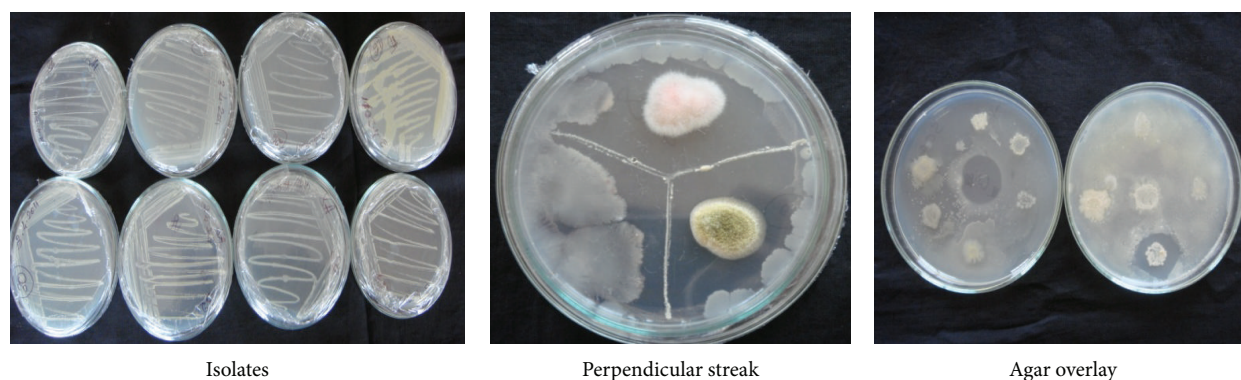


FIGURE 1: Pure culture of isolates and primary screening via streak plate assay along with agar overlay.

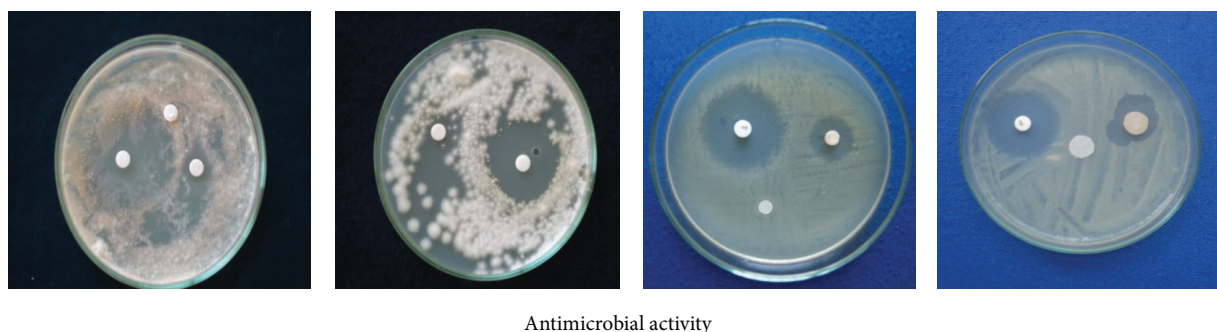


FIGURE 2: Antimicrobial activity of crude extract obtained from T28.

2.8. Partial Purification with HPLC. The crude ethyl acetate extract was partially purified using HPLC coupled with C-18 column. The mobile phase was with HPLC-grade water (component A) and acetonitrile (component B) in gradient mobile phase in linear mode consisting initially of A/B and increasing up to 100% B in 30 minutes at flow rate of 1 mL min^{-1} and injection volume at $10 \mu\text{L}$ with UV detector system at 220 nm. The eluting fractions were collected and visualized for band on TLC.

3. Results

Screening of *Tridax procumbens* L. for bacterial symbionts was successful with successive sterilization process. Use of cycloheximide during surface sterilization of leaves, stem, and roots segments suppressed the growth of endophytic fungi which resulted in the isolation of bacterial endophytes. The bacterial isolates were designated with alphanumeric codes T 01 to T 29 and preserved until further experiments. Isolates were dominant from surface sterilized stem with fourteen isolates identified from plant, six isolates from leaves, and nine isolates from roots, respectively. All the isolates exhibited myriad morphological characteristic and were subjected to preliminary screening which resulted in the inhibition of test fungal pathogens, namely, *F. verticillioides*, *A. niger*, and *A. flavus*, with perpendicular streak of isolates across the test pathogen (Figure 1). Agar overlay method for bacteria displayed clear zone of inhibition on the layer of test pathogenic bacteria laid over point-inoculated endophytic

bacteria (Figure 1). Based on the results ascertained from primary screening, one potent endophytic bacterium which displayed activity against all the test pathogens was selected as the subject of interest for large scale fermentation.

3.1. Evaluating Antimicrobial Activity. The crude ethyl acetate extract displayed antimicrobial activity by disc diffusion method against test pathogens with clear zone of inhibition across the disc (Figure 2). The obtained results showed the highest activity against *Shigella flexneri* (MTCC 731) followed by *Staphylococcus aureus* (MTCC 7443), and the least activity was conferred against *Xanthomonas campestris* (MTCC 7908) which was tabulated and interpreted with standards gentamicin and nystatin (Table 1).

3.2. TLC-Bioautography. Thin layer chromatography of ethyl acetate obtained from endophytic isolate T28 displayed separated bands at different R_f values with solvent system 1:1 hexane and ethyl acetate. The partially purified bioactive metabolite bearing antimicrobial activity showed prominent band at R_f 0.65 with clear zone of inhibition against test pathogen onto a red coloured background against MRSA strain as shown in Figure 3.

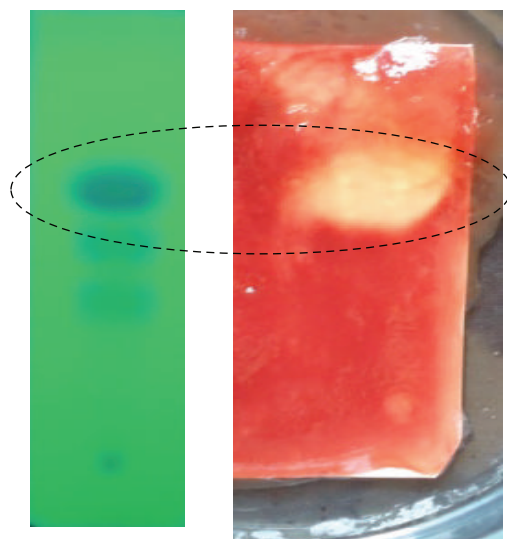
3.3. Partial Purification of Crude Extract. Partial purification of crude ethyl acetate extract was carried out with analytical technique HPLC; each fraction eluting was evaluated for antimicrobial potential upon which dominant peak bearing antimicrobial activity was eluted between 4 and 5 retention

TABLE 1: Antimicrobial activity of crude ethyl acetate extract against test pathogens.

Sl no.	Test pathogens	Ethyl acetate extract Zone of inhibition (mm)	Standard
I	Human pathogenic bacteria		
	<i>Bacillus subtilis</i> (MTCC 121)	13 ± 1.5	23 ± 1.5 (G)
	<i>Escherichia coli</i> (MTCC 7410)	12 ± 1.5	26 ± 1.5 (G)
	<i>Salmonella typhi</i> (MTCC 733)	18 ± 1.5	23 ± 1.5 (G)
	<i>Shigella flexneri</i> (MTCC 731)	27 ± 1.5	32 ± 1.5 (G)
	<i>Staphylococcus aureus</i> (MTCC 7443)	23 ± 1.5	31 ± 1.5 (G)
	<i>Staphylococcus epidermidis</i> (MTCC 435)	14 ± 1.5	20 ± 1.5 (G)
II	Plant pathogenic bacteria		
	<i>Xanthomonas campestris</i> (MTCC 7908)	10 ± 1.5	24 ± 1.5 (T)
III	Human pathogenic fungi		
	<i>Candida albicans</i> (MTCC 183)	16 ± 1.5	23 ± 1.5 (M)
IV	Plant pathogenic fungi		
	<i>Fusarium verticillioides</i>	14 ± 1.5	24 ± 1.5 (B)
	<i>Aspergillus niger</i>	10 ± 1.5	19 ± 1.5 (B)
	<i>Aspergillus flavus</i>	13 ± 1.5	22 ± 1.5 (B)

Values are zone of inhibition in mean ± standard error.

Note. G = gentamicin, T = tetracycline, M = miconazole, and B = Bavistin.



TLC-bioautography

FIGURE 3: TLC-bioautography of crude extract obtained from T28 against MRSA.

times (Figure 4) which was collected and verified resulting in activity against all the test pathogens.

4. Discussion

The results obtained in the present investigation are promising enough towards the ongoing efforts to combat multidrug resistant microorganisms. The use of plant origin products has served mankind since ancient era, but slow growing rate and harvesting of rare endangered species pose a risk and imbalance to the biodiversity of plants. Hence,

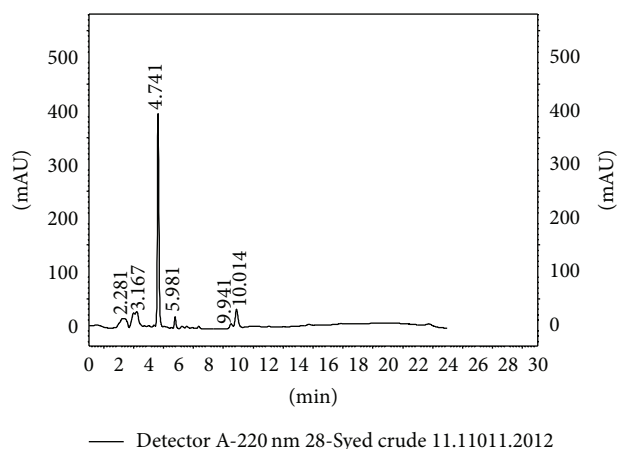


FIGURE 4: HPLC chromatogram of crude ethyl acetate extract.

scientific communities are reaching beyond plant kingdom for inexhaustible source of antimicrobial metabolites with economical feasibility and minimize the high cost [12]. Microbial flora form one of the largest diverse biological entities on the planet. Associations of these microbial entities with higher organisms have incremented their new roles and secondary metabolites with significant biological activities. One such plethora of microbial community is endophytic microorganisms which live as symbionts in almost every plant species. Research on endophytes has envisioned new interesting facets; hence, the present study was to screen and evaluate bacterial symbionts inhabiting *Tridax procumbens* L. for antimicrobial potentials. Screening of endophytes from plant reported to have medicinal value is one of the immense important criteria to discover potent endophytes as earlier reports confer that endophytes mimic the host

chemistry and secrete the bioactive compound similar to that of its host. Hence, in the present investigation, *Tridax procumbens* L. was selected for isolation of endophytes; earlier studies on endophytes from this plant are pertaining to fungal endophytes wherein the present study reports the evaluation of endophytic bacteria for antimicrobial potential [2]. In order to achieve potent endophytes, successful surface sterilization is critical factor. There are various surface sterilization protocols and different disinfectants such as sodium hypochlorite, mercuric chloride, ethanol, and calcium carbonate [13, 14]. But the majority of reports suggest the use of sodium hypochlorite coupled with ethanol for the isolation of endophytes [15–17], based on which sodium hypochlorite and ethanol were used in the present investigation, which resulted in the isolation of 29 bacterial endophytes. In order to screen for antimicrobial potential, each isolate was subjected for preliminary screening with agar overlay and streak plate techniques. These techniques are reported as accurate and rapid techniques which aid in screening large number of isolates [18–20]. Based on the results ascertained from preliminary screening, only one bacterium was selected, as it displayed activity against all the test pathogens compared to other isolates. The selected isolate T28 was subjected to large scale fermentation and organic phase was extracted with ethyl acetate as a solvent to obtain crude extract. Ethyl acetate forms one of the nondestructive, nontoxic, and nonpolar solvents with low boiling point compared to others and majority of the metabolites miscible with ethyl acetate [21]. Antimicrobial activity of crude extract was carried out using disc diffusion assay. Perusal of literatures suggests that antimicrobial metabolites usually produce in lesser quantity and often bound with other secondary metabolites in crude extract which may cause synergistic effect. Hence, in order to achieve the maximum activity, 50 μ L crude ethyl acetate extract (500 μ g/disc) from 10 mg/mL concentration was evaluated [22]. Significant activity was conferred against test bacteria compared to test fungus with the highest activity being displayed against *Shigella flexneri* which is a causative agent of *Shigellosis* followed by Methicillin resistant *Staphylococcus aureus*. Similar studies have been reported on the isolation of bacterial endophytes exhibiting antimicrobial potential [19, 23]. The active fraction of the crude extract was further partially purified with TLC and HPLC with bioautography assay, suggesting active band at R_f 0.65 bearing activity against test pathogens. The obtained results of HPLC and bioautography are congruent with earlier report of *Paenibacillus polymyxa* M-1, rhizobacterium exhibiting significant activity against *Erwinia* species.

5. Conclusion

The present study adds tremendous knowledge on the growing research towards developing new antimicrobial agents to combat drug resistant microorganisms. The study also contributes towards isolation of potent endophytes from *Tridax procumbens* L. which has scanty reports on their microbial symbionts. Significant activity against *Shigella flexneri* and Methicillin resistant *Staphylococcus aureus* suggests that the fraction is more potent as bactericidal agent compared

to antifungal. Future studies are highly recommended to characterize any novel metabolite responsible for activity.

Conflict of Interests

The authors declare that they have no conflict of interests.


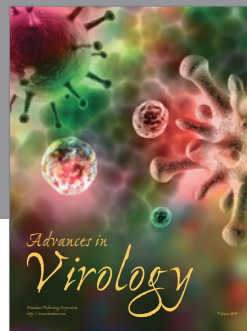
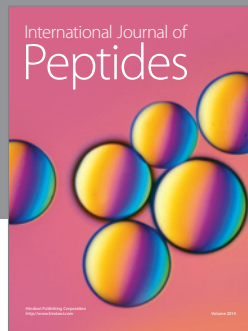
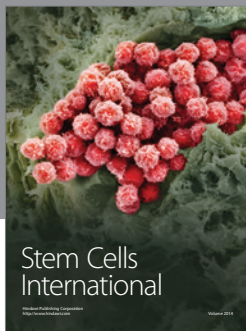
Acknowledgments

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